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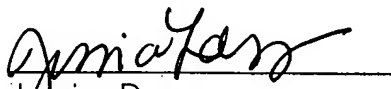
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**Method for Fluorescence Microscopy**

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The invention pertains to a method in fluorescence microscopy, especially laser scanning microscopy.

**Prior Art**

A conventional application of light microscopy for investigation of biological preparations is fluorescence microscopy (lit.: Pawley, "Handbook of biological confocal microscopy", Plenum Press, 1995). Specific dyes are used here for specific marking of tissues, cells or cell parts or also materials.

The emitted photons of a specific energy excite the dye molecules from the ground state to an excited state by absorption of a photon. This excitation is generally referred to as one-photon absorption (Figure 1a). The dye molecules so excited can return in different ways to the ground state. In fluorescence microscopy the transition with emission of a fluorescence photon is the most important. The wavelength of the emitted photon is generally red-shifted based on the Stokes shift in comparison to the excitation radiation and therefore has a greater wavelength. The Stokes shift permits separation of the fluorescence radiation from the excitation radiation.

A multiphoton excitation is shown in Figure 1b.

The fluorescence light is split and observed separately from the excitation radiation with appropriate dichroic beam splitters in combination with block filters. The depiction of individual cell parts stained with different dyes is possible on this account. However, several parts of a preparation, in principle, can also be simultaneously stained with different, specifically adding dyes (multiple fluorescence). Special dichroic beam splitters are again used to distinguish the fluorescence signals emitted by the individual dyes.

The prior art is to be explained below on the example of a confocal laser scanning microscope (LSM) (Figure 2).

An LSM is divided into essentially four modules: light source, scan module, detection unit and microscope. These modules are further described below. DE 19702753 A1 is additionally referred to.

For specific excitation of different dyes in one preparation, different wavelengths are used in an LSM laser. The choice of excitation wavelength is guided according to the absorption properties of the dyes being investigated. The excitation radiation is generated in the light source module. Different lasers are used here (argon, argon-krypton, TiSa laser). Selection of the wavelength and adjustment of the intensity of the required excitation wavelength additionally occurs in the light source module, for example by using an acousto-optic crystal. The laser radiation then goes to the scan module via a fiber or appropriate mirror arrangement.

The laser radiation generated in the light source is focused into the preparation with limited diffraction with objective (2) via the scanner, scan optics and tube line. The focus scans the

sample in the x-y direction point-like. The pixel residence times during scanning over the sample are generally in the range of less than a microsecond to a few seconds.

During confocal detection (descanned detection) of the fluorescence light, the light emitted from the focal plane (specimen) and from the overlying and underlying planes goes to a dichroic beam splitter (MDB) via the scanner. This separates the fluorescence light from the excitation light. The fluorescence light is then focused on a diaphragm (confocal diaphragm/pinhole), which is situated precisely in a plane conjugated to the focal plane. Fluorescence light fractions outside of the focus are suppressed on this account. By varying the diaphragm size the optical resolution of the microscope can be adjusted. Behind the diaphragm there is an additional dichroic block filter (EF) that again suppresses the excitation radiation. After passing through the block filter, the fluorescence light is measured by means of a point detector (PMT).

During use of multiphoton absorption, excitation of dye fluorescence occurs in a small volume on which the excitation intensity is particularly high. This region is only slightly larger than the detected region during use of a confocal arrangement. The use of a confocal diaphragm can therefore be omitted and detection can occur directly after the objective (nondescanned detection).

In another arrangement for detection of dye fluorescence excited by multiphoton absorption descanned detection again occurs, but this time the pupil of the objective is imaged in the detection unit (nonconfocal descanned detection).

Only the plane (optical section) that is situated in the focal plane of the objective is detected by a three-dimensionally illuminated image by both detection arrangements in conjunction with the corresponding one photon or multiphoton absorption. By marking several optical sections in the x-y plane at different depths z of the sample, a three-dimensional image of the sample can then be generated in computer-controlled fashion. The LMS is therefore suitable for investigating thick preparations. The excitation wavelengths are determined by the employed dye with specific absorption properties. Dichroic filters adjusted to the emission properties of the dye ensure that only the fluorescence light emitted by the corresponding dye is measured by the point detector.

In biomedical applications several different cells or cell regions are now marked with different dyes simultaneously (multifluorescence). The individual dyes can be detected separately with the prior art based either on different absorption properties or emission properties (spectra). For this purpose additional splitting of the fluorescence light from several dyes occurs with the secondary beam splitters (DBS) and a separate detection of the individual dye emissions in separate point detectors (PMT x). Flexible adjustment of detection and excitation to corresponding new properties by the user is not possible with the arrangement described above. New dichroic beam splitters and block filters must instead be created for each (new) dye.

If the emission spectra of two dyes overlap, the previous detection devices reach their limits. In order to avoid overlap between two dyes, the spectral detection range must be restricted. The range in which the two dyes overlap is simply cut out for this purpose and not detected. The efficiency of the detection unit therefore deteriorates. An equal signal-to-noise ratio can only be achieved by increasing the excitation power, through which preparation damage can occur.

Nowadays a maximum of up to six different dye probes are therefore simultaneously used, since the dyes otherwise could not be separated owing to the strongly overlapping emission bands.

Previously dyes have been modified so that they either differ from each in their absorption properties or in their emission properties. Figure 3 shows the emission spectra of different typical dyes. The emission signal is plotted as a function of wavelength. The dyes denoted 1 to 4 differ in position and form of their emission spectra. These dyes, however, are in most cases toxic for living preparations. Investigations on evolution of cell structure in living preparations are therefore impossible. In the late 90s, dyes occurring in nature, the so-called fluorescing proteins (GFP, YFP, CFP, TOPAS, GFT, RFP) were discovered (company: Clontech, USA).

Fluorescence dyes for specific marking of preparations are used in all of the aforementioned systems.

In the Zeiss laser scanning microscope META the fluorescence is split spectrally. For this purpose the emission layer is split from the excitation light in the scan module or in the microscope (during multiphoton absorption) by means of the main color divider (MDB). A block diagram of the following detector unit is shown in Figure 5. The light of the sample is focused by means of an imaging optics PO during confocal detection through an iris (pinhole) PH so that fluorescence that originated outside of the focus is suppressed. During nondescanned detection the diaphragm drops out. The light is now broken down into its spectral fractions by means of an angle-dispersive element DI. Prisms, gratings and acousto-optic elements are considered as angle-dispersive elements. The light split by the dispersive element into its spectral components is finally imaged in a line detector DE. This line detector DE therefore measures the emission signal as a function of wavelength and converts it to electrical signals. In addition, the detection unit can have a line filter connected in front to suppress excitation wavelengths.

The depicted structure essentially describes a Cerny Turner design. During confocal detection the light L of the sample is focused with the pinhole optics PO through the confocal diaphragm PH. During a nondescanned detection in the case of multiphoton absorption, this diaphragm can drop out. The first imaging mirror S1 collimates the fluorescence light. The light then impinges on a line grating G, for example a grating with a line number of 651 lines per mm. The grating diffracts the light according to its wavelength in different directions. The second imaging mirror S2 focuses the individual spectrally split wavelength fractions onto the corresponding channels of the line detector DE. The use of a line-secondary electron multiplier of the Hamamatsu Co. H7260 is particularly advantageous. The detector has 32 channels and high sensitivity. The free spectral range of the aforementioned variant is about 350 nm. The free spectral range in this arrangement is uniformly distributed in the 32 channels of the line detector so that an optical resolution of about 10 nm is produced. This arrangement is only conditionally suitable for spectroscopy. However, its use in an imaging system is advantageous, since the signal is still relatively large per detection channel because of the relatively broad detected spectral band. A shift of the free spectral region can additionally occur by rotation of the grating.

Another possible variant could involve the use of a matrix detector (for example CCD). In this case splitting into different wavelength fractions is carried out in one coordinate by the dispersive element. In the remaining direction a complete line (or column) of the scanned image is imaged on the matrix detector. This variant is particularly advantageous in constructing a line scanner (lit.: Corle, Kino: "Confocal Scanning Optical Microscopy and Related Imaging Systems," Academic Press, 1996). The essential design corresponds essentially to that of an LSM according to Figure 2. However, instead of a point focus, a line is imaged in the focus and the sample being investigated is only scanned in one direction. A slit diaphragm instead of a perforated diaphragm serves as confocal diaphragm in such a structure. A nondescanned detection during use of multiphoton absorption can also occur with this arrangement. The confocal diaphragm can again drop out for this purpose.

By spectral splitting of the fluorescence light, after recording of the fluorescence spectra of the fluorescence markers in pure form and recording of the spectra with fluorescence fractions of several markers, a separate recording of the spectral fractions can occur by an unmixing method (DE 19915137 A1).

**Invention:**

It has now been recognized according to the invention that the number of fluorescence markers used for fluorescence marking can be reduced or combinatorics utilized, if not only fluorescence spectra are to be used in pure form as reference spectra but also reference spectra of mixed forms are recorded. These mixed forms can be characterized, for example, by the time-dependent color state of the biological material, if a fluorescence marker slowly leads to discoloration.

In addition, such mixed states can be characterized by a mixed color if a fluorescence marker changes its color or its excitation properties.

Such mixing conditions can be produced in different ways: they can be present in the sample, be produced by irradiation of the sample or be the result of a biological process excited by irradiation.

Mixed spectra can characterize a biological process, for example a concentration change, in which a first spectrum corresponds to a lower concentration state and at least one additional spectrum corresponds to a higher concentration state.

By means of the different reference spectra, image channels are defined and correspondingly evaluated. The generation of such references can occur over the entire image or advantageously over marked "regions of interest" (ROI). A deliberate manipulation by defined irradiation can also occur over such ROI. A reference can also be determined in a first region and deliberate irradiation and measurement can occur in an additional region by extraction of mixed spectra. Demixing and depiction of the spectra can occur after imaging recording or during imaging recording.

It was surprisingly found that new markers like the fluorescing protein Kaede, which turns from green to red during irradiation (lit.: Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. and Miyawaki, A. (2002), An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein, PNAS 99/20, 12651-12656) during recording of organic processes, for example inter- and intracellular processes can be used for marking of individual cells or cell populations and spectrally detected. Photoconvertible dyes that change their spectra dynamically based on intracellular processes or dyes that are used for FRET, but also other indicator dyes can be advantageously used by the method according to the invention.

For example, different cells or cell groups in a cell population that has been marked with the dye Kaede can be exposed and converted for different lengths of time with UV or violet light. Different color mixing ratios are then established that are recorded as reference spectra. Different cell populations can then be individually recorded over time. This is true not only for cell cultures, but can also concern both subcellular structures and also entire organisms, in which a certain cell population is irradiated and can be observed in its development, in which the resulting reference can be assigned to specific image channels and can therefore be followed.

A corresponding region can be selected over the ROI. An analysis of transport processes on the cellular and subcellular level can occur. Advantageously, still only one dye is used, which is

placed in different states via radiation of other effects that are clearly identifiable by reference formation.

A different rise in fluorescence intensity, as during PA-GFP (photoactivatable GFP, lit.: Patterson, G. H. and Lippincott-Schwartz, J. (2002), A photoactivatable GFP for selective photobleaching of proteins and cells, *Science* 297, 1873-1877) after excitation with violet light can serve as reference. In the Zeiss LSM META ROIs can be interactively defined directly in the image. The selected laser is switched on and off with pixel precision at the boundary of these regions.

In time series dialog the start and end of the time series as well as intervals and delays between recordings are fixed. The irradiation parameters that lead to a change in dye properties by photoactivation or photoconversion, for example, repetition rate, wavelength, intensity, position can be automatically incorporated. Evaluation can occur after the experiment or on-line during recording in order to be able to intervene directly in the course of the experiment. The average intensities of ROI in time, as well as the times of photoactivations and conversions are indicated.

The META detector permits the entire spectrum of emission, for example of Kaede, to be recorded and to separate the corresponding mixed forms spectrally during measurement and to display the demixed channels.

Figure 4 schematically depicts how different image channels CH1-CH3 are formed, in which, as shown, different spectral mixed distribution of CH1-3 are used as reference and referred to for image evaluation.

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**Patent Claims**

1. Method for fluorescence microscopy, especially with a laser scanning microscope in which an at least partially spectrally resolved detection of the fluorescence spectrum occurs and reference spectra are used for spectrally demixing, characterized by the fact that recording of the reference spectra occurs from temporally and/or spectrally variable dyes and/or dye combinations and serves for image evaluation.
2. Method according to Claim 1 for recording of organic processes.
3. Method according to Claim 2 for recording of intracellular processes.
4. Method according to Claim 2 for recording of intercellular processes.
5. Method according to one of Claims 1 to 4 for recording of cells and/or cell populations.
5. Method according to one of Claims 1 to 5 in which recording of reference spectra of photoconvertible dyes occurs.
7. Method according to one of Claims 1 to 7 in which recording of reference spectra of photoactivatable dyes occurs.
8. Method according to one of Claims 1 to 7 in which recording of reference spectra of indicator dyes occurs.
9. Method according to one of Claims 1 to 8 in which recording of reference spectra of dyes occurs that change their spectra dynamically based on intracellular processes.
10. Method according to one of Claims 1 to 9 in which recording of reference spectra of dyes occurs with a different rise in fluorescence intensity.
11. Method according to one of Claims 1 to 10 in which recording of reference spectra of the fluorescing protein Kaede occurs.
12. Method according to one of Claims 1 to 11 in which recording of reference spectra of PA-GFP occurs.